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(54) Title: MANUFACTURING PROCESS FOR LIPOSOMAL PREPARATIONS

(57) Abstract: The present invention provides manufacturing processes for liposomal preparations. In accordance with the methods, a lipid fraction is dissolved in a water-miscible organic solvent. This solution comprising the lipid fraction can be added to and mixed with an aqueous solution under conditions to form a bulk liposomal preparation. Desirably, the preparation can include one or more active principals. The bulk liposomal preparation can be further processed as desired, for example, by size fractionation or reduction, removal of the water-miscible organic solvent, freeze-drying, or other treatment. The methods permit the production of liposomal formulations on a large or commercial scale.

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MANUFACTURING PROCESS FOR LIPOSOMAL PREPARATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to co-pending United States Provisional Patent Application 60/446,895, filed on February 11, 2003, the entirety of which is incorporated herein by reference thereto.

TECHNICAL FIELD OF THE INVENTION

[0002] The present invention relates to methods of manufacturing a liposomal preparation and the liposomal preparation produced by these methods.

BACKGROUND OF THE INVENTION

[0003] Many known methods exist for manufacturing liposomal formulations of various active principals (typically antineoplastic agents, antifungal agents, and the like). Such methods include, for example, ethanol dilution, thin film hydration, the methylene chloride process, and the like.

[0004] Other methods have been proposed involving t-butanol to dissolve liposome-forming – lipids to manufacture dried lipid powders by lyophilization (see, e.g., U.S. Patents 6,146,659 and 6,090,407). Hydration of the dried lipid powders with suitable aqueous media results in the formation of multi-lamellar liposomes that are size reduced by sonication and nebulization for administration. T-butanol has not been used as the primary choice solvent for manufacturing liposomes, however, mainly due to: i) its limited lipid solubility (cholesterol in particular) in t-butanol. ii) its acceptability as a pharmaceutical excipient in parenteral dosage forms. iii) the necessity for its removal upon liposome formation.

[0005] While effective on a small-scale basis, current methods generally are unsuitable for manufacturing large quantities of liposomal formulations of many active principals, particularly paclitaxel and other anticancer agents. As a result, current methods for manufacturing liposomal formulations are not adequate to supply commercial quantities of liposomal preparations of many pharmaceutical agents. Thus, there is a need for a process for manufacturing liposomal formulations of active principals that can be employed on a large or commercial scale.

SUMMARY OF THE INVENTION

[0006] The present invention provides manufacturing processes for liposomal preparations. In accordance with one aspect of the inventive method, a lipid fraction is dissolved in a water-miscible organic solvent. This solution comprising the lipid fraction can be added to and mixed with an aqueous solution under controlled conditions suitable to form a bulk liposomal preparation.

[0007] Desirably, the preparation can include one or more active principals. In accordance with another aspect of the inventive method, at least one active principal and a lipid fraction are dissolved in a water-miscible organic solvent. This solution comprising the active principal and lipid fraction can be added to and mixed with an aqueous solution under controlled conditions suitable to form a bulk liposomal preparation.

[0008] The bulk liposomal preparation can be further processed as desired, for example by size fractionation or reduction, removal of the water-miscible organic solvent, sterilization by membrane filtration, freeze-drying, or other treatment.

[0009] The invention further provides a liposomal preparation produced by the manufacturing processes of the present invention and methods of using such formulations.

[0010] The invention permits the production of liposomal formulations on a commercial scale. These advantages of the present invention, and additional inventive features, will be apparent from the following detailed description and the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Figure 1 is a histogram presenting the size distribution of paclitaxel containing liposomes prepared by t-butanol process after size reduction.

[0012] Figure 2 is a flow chart for solvent removal by tangential flow filtration.

[0013] Figure 3 is a histogram presenting the size distribution of paclitaxel containing liposomes prepared by t-butanol process after size reduction and solvent removal by tangential flow-filtration.

[0014] Figure 4 is a histogram presenting the size distribution of paclitaxel containing liposomes (reconstituted after freeze drying) prepared by t-butanol process.

[0015] Figure 5 is a freeze fracture electron micrograph of paclitaxel containing liposomes (reconstituted after freeze drying) prepared by t-butanol process.

DETAILED DESCRIPTION OF THE INVENTION

[0016] In accordance with the inventive methods, a water-miscible organic solvent is employed to dissolve a lipid fraction and/or one or more active principals. Many such water-miscible organic solvents (e.g. dimethylsulfoxide, ethanol, and methanol) can be used in the context of the present invention. However, the most preferred water-miscible organic solvent is t-butanol.

[0017] The lipid fraction can comprise any suitable lipid or lipids of which it is desired to form liposomes. Preferred lipids in the lipid fraction include, for example, one or more of cholesterol, dioleoylphosphatidylcholine (DOPC), tetramyristoyl cardiolipin, and tocopheryl acid succinate. In some embodiments, tetramyristoyl cardiolipin can be substituted with positively charged cationic cardiolipins, such as 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propan-2-ol [*(R)*-PCL-2] and the like. Preferably, the lipid fraction includes an antioxidant, such as tocopheryl acid succinate. More preferably, the lipid fraction includes at least two (such as three or more) of these compounds, and most preferably the lipid fraction includes this entire group of compounds. Depending on the desired composition of the lipid fraction, the amount of the various lipids can be adjusted as desired. However, a preferred composition of the lipid fraction includes a majority of the lipids as DOPC, for example a DOPC:Chol:Cardiolipin 90:5:5 molar ratio. Where an antioxidant is included, a suitable molar ratio is 89:5:5:1 DOPC:Chol:Cardiolipin:Tocopheryl acid succinate.

[0018] In some embodiments, an effective formulation can be produced by sequential addition or dissolution of the lipids that form the lipid fraction in the water-miscible organic solvent. Most preferably, the method involves sequential addition of cholesterol, DOPC, tetramyristoyl cardiolipin, and tocopheryl acid succinate so as to dissolve each into the water-miscible organic solvent. In many embodiments, it is desirable for the lipid fraction to be dissolved in the water-miscible organic solvent at temperatures above room temperature (i.e., about 25 °C). Thus, where t-butanol is the desired solvent, the lipids can be added at temperatures between about 35 °C and about 65 °C, such as between about 45 °C and about 55 °C.

[0019] After the lipid fraction is added to the water-miscible organic solvent, the resulting solution can be added to an aqueous solution to form a bulk liposome preparation. At this stage, the bulk liposome preparation typically comprises multilamellar liposomes, as assessed, for example, by dynamic light scattering.

[0020] For forming the bulk liposome preparation, the amount of aqueous solution can vary, but generally it is a majority of the batch size, i.e., the volume of the total liposome preparation. Preferably, the amount of aqueous solution is at least about 80% of batch size, and the amount of aqueous solution more preferably is at least about 90% of batch size. In some embodiments, the amount of aqueous solution can be more than the batch size.

[0021] The aqueous solution can be water but more typically contains one or more additional ingredients, such as sugars, tonicity adjusters, and the like. Suitable tonicity adjusters include salts (preferably sodium chloride) and other agents known to those of ordinary skill in the art. Tonicity adjusters can be present in any suitable amount; however, when present, the tonicity adjusters typically represent less than about 2% of the aqueous solution, and more typically less than about 1% of the aqueous solution. Preferably, the aqueous solution contains a protective sugar (such as, for example, trehalose, sucrose, maltose, lactose, glucose, dextran, etc., as well as combinations of these). One or more of such protective sugars can be present in any suitable amount. However, when present, the protective sugar(s) adjusters typically represent at least about 5% of the solution, and generally less than about 20% of the aqueous solution (more typically less than about 15% of the aqueous solution). A most preferred aqueous solution for this purpose is 10-12% sucrose and 0.4-0.9% sodium chloride.

[0022] The water-miscible organic solvent solution containing the lipid fraction can be added to the aqueous solution by any method able to achieve the formation of the bulk liposome preparation. However, allowing the lipid solution in t-butanol to cool below 40°C results in lipid precipitation. Likewise, addition of lipid solution to aqueous phase solution maintained at room temperature also can result in precipitation of lipid and (when present) active principal. Accordingly, it is preferable for the water-miscible organic solvent solution to be added to the aqueous solution with mixing (e.g., using a conventional mixer, such as those manufactured by Labmaster), for example at between about 300 rpm to about 400 rpm, while maintaining the temperature above 30 °C, such as maintaining the aqueous solution at between about 30°C and about 40°C. Also, it often will assist the formation of liposomes for the water-miscible organic solvent solution containing the liposomal fraction to be added to the aqueous solution while maintaining the temperature about 35 °C. For example, when added to the aqueous solution, the water-miscible organic solvent can be maintained between about 25 °C and about 40 °C, more preferably between about 30 °C and about 40 °C, and most preferably between

about 30 °C and about 35 °C, particularly where the water-miscible organic solvent is t-butanol.

[0023] The rate at which the water miscible organic solvent containing lipid fraction is added to the aqueous solution and the rate of mixing of aqueous solution during such addition manifest the formation of liposomes containing the lipid soluble active principal (paclitaxel, docetaxel) without precipitation. For example, where t-butanol serves as the water-miscible organic solvent, it and the aqueous solutions can be combined while mixing for between about 5 minutes and about 1 hour, more typically between about 10 minutes and about 45 minutes, and typically between about 15 minutes and about 30 minutes. For large-scale production, the duration of addition (i.e., period of mixing) can be considerably longer, such as several hours or more. Also, the mixing speed can be somewhat less than 300 rpm or somewhat more than 400 rpm, as noted above, as needed, such as, for example, at least about 200 rpm or at least about 500 rpm and up to about 800 rpm or even up to about 1000 rpm. Thus, the mixing speed can be between about 200 rpm and about 800 rpm, such as between about 500 rpm and about 1000 rpm. For large scale production, a preferred range is between about 600 rpm and about 800 rpm.

[0024] Alternatively, the addition of the water-miscible organic solvent solution comprising the lipid fraction to the aqueous solution can be accomplished while the solution is cooling. Typically, this involves mixing of solution following addition of water-miscible solvent comprising the lipid fraction to the aqueous solution while cooling. For example, the water-miscible organic solvent solution with the lipid fraction can be added to the aqueous solution while cooling to a temperature between about 25 °C and about 30 °C.

[0025] In many applications it is desirable for the liposomal preparation to be used in medical applications. For such applications, the preparation can contain one or more active principals. An active principal can be any agent (or combination of agents) desired to be formulated into a liposomal preparation, such as a small molecule, oligonucleotide, or other agent. Typically, the active principal includes at least one antineoplastic or antifungal agent. Preferred active principals are agents such as taxanes or derivatives, such as paclitaxel, docetaxel, and related compounds (e.g., epothilones A and B, epothilone derivatives, etc.) and other anticancer agents such mitoxantrone, camptothecins, and related molecules (such as, for example, 7-ethyl-10-hydroxycamptothecin (i.e., SN-38), irinotecan, etc.) and derivatives, doxorubicin, daunorubicin, methotrexate, adriamycin, tamoxifen, toremifene, cisplatin, epirubicin,

gemcitabicine HCl, mixotantrone, and other known chemotherapeutics useful for treatment of cancer and antisense oligonucleotide (such as antisense oligonucleotides that inhibit the expression of an oncogene, see, e.g., U.S. patents 6,559,129, 6,333, 314, and 6,126,965, disclosing a 15-mer anti c-raf-1 oligonucleotide having the sequence 5'-GTGCTCCATTGATGC-3').

[0026] Preferably the active principal comprises at least one agent selected from the group consisting of taxanes or derivatives and camptothecin or derivatives. One of ordinary skill in the art will recognize that derivatives or analogues will have the same activity as the unaltered agent, optionally to a greater of lesser extent, but not negated. Such chemical modifications will be based on structure activity relationships (SAR) or molecular modeling. For example, functional groups can be substituted or eliminated. A most preferred active principal is paclitaxel.

[0027] While any amount of active principal can be employed, as desired, where paclitaxel is used, typically an amount of active principal of at least about 1% weight, relative to the batch size, is dissolved in the water-miscible organic solvent. More typically, at least where 1 mg/ml paclitaxel (relative to batch size) is employed, the paclitaxel is dissolved in at least about 5% by volume of t-butanol, relative to batch size. It is possible, in some embodiments, for the amount of active principal to exceed about 5% by volume, relative to batch size. In the same manner, up to 10% by volume t-butanol or a mixture of t-butanol and ethanol not exceeding 1:1 (volume ratio) and a total of 10% by volume may be used.

[0028] The one or more active principals are added during the formulation process in a manner appropriate to the chemistry of the compound. For example, water-soluble principals (e.g., antisense oligonucleotides) can be added to the aqueous solution, such as before bulk liposome formation. The addition of the water-soluble principals to the aqueous solution can be prior to the addition of the water-miscible organic solvent so as to be entrapped in the liposomes or bound to the liposomes. Alternatively, some water-soluble active principals (e.g., SN38) can be added to the size reduced liposomes after solvent removal but before sterile filtration and freeze-drying, steps which are described below. Further, active principals, such as ones that are soluble in organic solvents, can be added by dissolving them in the water-miscible organic solvent. Preferably, the active principals soluble in organic solvents can be added to the water-miscible organic solvent prior to the addition of the lipid fraction. The one or more active principals can be added

during or after mixing the water-miscible organic solvent solution comprising the lipid fraction with the aqueous solution.

[0029] In many embodiments, it is desirable for the one or more active principals (excluding water soluble agents) to be dissolved in the water-miscible organic solvent, particularly t-butanol, at temperatures above room temperature (e.g., about 35 °C). Thus, for example, where paclitaxel is the desired active principal, it can be dissolved in a water-miscible organic solvent, such as t-butanol, at temperatures between about 35 °C and about 65 °C, such as between about 40 °C and about 55 °C. The temperature at which other active principals can be dissolved in t-butanol or in other water-miscible organic solvents may vary depending on the properties of the active principals, but it is within the ordinary skill of the art to select a suitable temperature for dissolution. As mentioned above, it often is desirable for the water-miscible organic solvent solution containing the lipid fraction and the active principal to be added to the aqueous solution while maintaining the temperature.

[0030] It is often preferred that the bulk liposome preparation formed by these methods be size reduced or fractionated or otherwise controlled. Such a sizing treatment is preferably applied to render the particle size of the liposomes more uniform. The mean size of the liposome formulation can be, for example, about 50 nm to about 200 nm, preferably 100-180 nm, and more preferably 100-160 nm as measured by dynamic light scattering techniques. In addition, 99 percentile distribution (D99) of the size reduced liposomes can be, for example, about 100 nm to about 400 nm, preferably 150-300 nm, more preferably 180-250 nm as measured by dynamic light scattering techniques. An exemplary way to achieve this is to treat the bulk liposome preparation by extrusion through a sieve, such as a polycarbonate filter, of a pre-selected size (such as 0.2 μm, 0.1 μm, etc.). Preferably, the liposomes are size reduced by extrusion through 0.2 μm and 0.1 μm polycarbonate filters at pressures typically up to about 200 psi without precipitation of any active principal from the preparation. For larger scale production, the pressure can be expanded beyond about 200 psi, such as between about 200 psi and about 800 psi.

[0031] The bulk liposome preparation (or the size-reduced preparation) will contain most of the water-miscible organic solvent employed initially to dissolve the lipid fraction. For many applications, principally medical uses, it is desirable to substantially remove the solvent (and more desirably, completely remove the solvent) from the bulk or size-reduced liposome preparation. Furthermore, if the preparation is to be freeze dried,

it is essential to substantially remove (preferably completely remove) the water-miscible organic solvent, t-butanol, to preserve liposome size and maintain active principal in the liposomes during the freeze drying process. One preferred method of substantially freeing the liposome preparation from water-miscible organic solvent (particularly t-butanol) involves diafiltration using a tangential flow filtration process.

[0032] As an example, size reduced liposomes can be recirculated through nominal molecular weight cut-off (MWCO) (ranging from 10,000 Daltons to 500,000 Daltons) membrane filter cassette or cartridge with surface areas ranging from 0.1 sq. meters to several hundred sq. meters that permit the passage of small molecules with less than 1000 Daltons. Recirculation of liposome solution through these membrane filters and by way of restricting the outlet flow, transmembrane pressure can be generated against the pores in the membrane allowing small molecules (e.g., 10% sugar solution and t-butanol) to pass through. This procedure can be performed either in continuous mode or concentration mode. In continuous mode, aqueous phase used in preparing the liposomes is added to the recirculating liposomes at the same rate as the filtrate is removed. In concentration-dilution mode, aqueous phase containing t-butanol is removed from the size reduced liposomes, thus concentrating the liposome solution to a desired volume, preferably 50% of the initial volume, and then adding aqueous phase used in preparing the liposomes to return back to starting volume. This procedure can be repeated in an iterative manner until the water-miscible solvent (e.g., t-butanol) is removed to desired levels, preferably less than 1% of the total volume. In either continuous or concentration-dilution mode, a minimum of four volumes (initial starting volume) of aqueous phase is exchanged to remove t-butanol to acceptable levels.

[0033] Sterile filtration of liposomal products is an alternate to conventional sterilization procedures (terminal heat sterilization such as autoclaving, gamma radiation, and ethylene oxide treatment), which is a prerequisite (regulatory requirement) for all parenteral dosage forms of medicinal application. By way of passing the liposomes through a sterile 0.22 μ filter, all viable microbes are removed from the liposome product. Sterile filtration is performed prior to filling the product in sterilized containers under aseptic conditions.

[0034] Following production, and (if desired) size-control and/or removal of water-miscible organic solvent, the bulk or size-reduced lipid preparation preferably is freeze-dried. Any suitable device or method can be employed. A preferred device is a Genesis - 25EL (manufactured by Virtis) and any suitable size lyophilizer (e.g., such as those

manufactured by Virtis, Edwards, and Hull Corp.). The bulk or size-reduced liposome preparation can be maintained in lyophilized form (e.g., in cold storage at about –2-8 °C,) for an extended period of time, such as for at least about several months or years.

[0035] For use, the lyophilized bulk or size-fractionated liposomal preparation can be reconstituted with a suitable volume of reconstitution solution, which preferably is a polar solvent, and most preferably an aqueous system, which can be de-ionized water or sterile water or a suitable aqueous saline solution. Any suitable volume of reconstitution solution can be employed, such as between about 1 ml and about 50 ml, more typically between about 3 ml and about 25 ml. For use, the liposomal formulation can be diluted as desired, such as in a suitable physiologically-compatible buffer or saline solution. To assist in reconstitution, the preparation can be mixed gently or vigorously agitated (snapping motion using thumb and index finger) as desired.

[0036] The invention further provides a liposomal preparation produced by the manufacturing processes as described herein and methods of using such formulations. The inventive liposomal preparation typically can be formulated for administration to a human or animal patient. For such uses, the inventive formulation can include, in addition to liposome formulations of active agents non-toxic, inert pharmaceutically suitable excipients. Pharmaceutically suitable excipients include solid, semi-solid or liquid diluents, fillers and formulation auxiliaries of all kinds. Tablets, dragees, capsules, pills, granules, suppositories, solutions, suspensions and emulsions, pastes, ointments, gels, creams, lotions, powders and sprays can be suitable pharmaceutical preparations. Suppositories can contain, in addition to the liposomal active agent, suitable water-soluble or water-insoluble excipients. Suitable excipients are those in which the inventive liposomal active agent is sufficiently stable to allow for therapeutic use, for example polyethylene glycols, certain fats, and esters or mixtures of these substances. Ointments, pastes, cream, and gels can also contain suitable excipients in which the liposomal active agent is stable. It is within the ordinary skill in the art to formulate liposomal preparations depending on the desired manner of application (e.g., parenterally, topically, orally, etc.)

[0037] The invention also includes pharmaceutical preparations in dosage units. This means that the preparations are in the form of individual parts, for example vials, syringes, capsules, pills, suppositories, or ampoules, of which the content of the liposome formulation of active agent corresponds to a fraction or a multiple of an individual dose. The dosage units can contain, for example, 1, 2, 3, or 4 individual doses, or 1/2, 1/3, or

1/4 of an individual dose. An individual dose preferably contains the amount of active agent which is given in one administration and which usually corresponds to a whole, a half, a third, or a quarter of a daily dose.

[0038] The inventive formulations, especially those that contain active agents, facilitate a method of treating a disease in a vertebrate (such as a human or non-human animal), comprising the step of administering a pharmaceutical preparation as described herein, which typically includes a therapeutic agent specific for the treatment of the disease, to the patient. In accordance with the inventive method, a preparation as herein described (desirably containing an active agent) is administered to a vertebrate in need of treatment in an amount and at a location sufficient to treat the disease within the vertebrate. The pharmaceutical preparation is administered to the patient in the manner appropriate to the type of formulation, such as intravenously, subcutaneously, locally, topically (e.g., to skin or dermal tissue, or to mucosal tissue), orally, parenterally, intraperitoneally, rectally, by direct injection into tumors or sites in need of treatment, etc. by such methods as are known or developed.

[0039] In one embodiment, the method disease is cancer, in which instance, the pharmaceutical preparation can comprise a suitable anticancer agent, such as herein described. In another embodiment, the disease is an infection, such as a viral, bacterial, or fungal infection. It should be realized that the effective treatment of a disease, in accordance with the inventive methods, while desirably eliminates the disease or its symptoms, need not completely eradicate the effects of the disease. Indeed, successful therapy in accordance with the inventive method can be measured by a reduction in the severity of a disease, infection, or a reduction in the rate by which a disease progresses within a patient.

EXAMPLE

[0040] The example demonstrates the manufacturing process for liposomal preparations of the present invention. This example is provided as a further guide to the practitioner of ordinary skill in the art and not to be construed as limiting the invention in anyway.

Materials and Methods:

[0041] In the example detailed below, DOPC, cholesterol, and tetramyristoyl cardiolipin were obtained from Avanti Polar Lipids, Inc., Alabaster, AL. Paclitaxel was

obtained from Hande Tech, Austin, TX; t-butanol and ethanol from J.T. Baker; sucrose from Mallinckrodt; and D-alpha tocopheryl acid succinate from Sigma.

[0042] Liposome Size measurements were made using Partcile Sizing Systems (PSS, CA) Z-380 instrument. Lyophilization was carried out using Genesis 25-EL (manufactured by VirTis). Pellicon 2 Tangential Flow Filtration system and the 100 kD MWCO polyether sulfone membrane cassettes were obtained from Millipore Corporation, Bedford, MA.

[0043] For freeze fracture electron microscopy, the sample was quenched using a sandwich technique in liquid nitrogen cooled propane at a cooling rate of 10,000 Kelvin per second to avoid ice-crystal formation and artifacts during cryo-fixation process. The cryo-fixed sample was fractured using a JEOL-JED-9000 freeze etching equipment and the exposed fracture planes were shadowed with platinum for 30 seconds at an angle of 25-35° and coated with carbon for 35 sec. The replicas were cleaned and examined using Philips CM 10 electron microscope.

Preparation of liposomal paclitaxel formulation with t-butanol solvent by the inventive method:

[0044] 200 ml and 500 ml batches of liposomal paclitaxel formulation at 1 mg/ml of the active principal were prepared as described below by the inventive method. For the purpose of this example, the process is described in detail with 200 ml batch as an example.

Table 1 lists the formulation composition and the batch quantities used in the preparation.

Table 1

Formulation composition and batch quantities for liposome based paclitaxel formulation using t-butanol

Chemical	Quantity * (mg/ml)	Batch Quantity
DOPC	27.00	5.40 g
Cholesterol	0.75	0.15 g
Tetramyristoyl Cardiolipin	2.45	4.90 g
Tocopheryl acid succinate	0.31	0.06 g
Paclitaxel	1.0	0.20 g
t-Butanol **	0.05 ml	8.0 g
10% Sucrose solution in normal saline	Q.S. to 1.04 g	208 g

* Final intended concentration of the ingredients in the formulation

** Specific gravity 0.789g/mL. To be removed during the process

[0045] 8.0 g of t-butanol solvent (after maintaining the solvent container at 35-40 °C) was first weighed in to pre-tared beaker with a stir bar.

[0046] 200 mg of paclitaxel was weighed and added to t-butanol with mixing while maintaining the solvent temperature above 35 °C by heating on a hotplate. The beaker was covered with an aluminum foil to prevent evaporative loss of solvent.

[0047] After paclitaxel was completely dissolved (duration about 15-20 min), 150 mg of cholesterol was weighed separately and added to t-butanol solution containing paclitaxel and mixed until completely dissolved (duration 3-5 minutes).

[0048] 490 mg of tetramyristoyl cardiolipin, 5.4 g of DOPC, and 62 mg of tocopheryl acid succinate were weighed individually and added in that order to t-butanol solution and mixed until the solution was free of any undissolved lipid while maintaining the solution temperature between about 40-50° C. Total duration for dissolution of all added components into solution was about 45 min, and the solution temperature was about 48 °C.

[0049] The aqueous phase solution of 10% sucrose and 0.9% sodium chloride (4000 ml) was prepared by dissolving 400 g of sucrose and 36 g of sodium chloride in deionized water (Milli Q systems) and the solution was filtered through MilliPak 20 sterilizing filter.

[0050] 190 g of the filtered sucrose solution was weighed into a pre-tared jacketed glass container and fitted with a circulating water bath set at 36 °C to maintain the temperature.

[0051] The sucrose solution was mixed using a Labmaster lightnin mixer at 300 rpm for 10 minutes to equilibrate the solution temperature to 35 °C.

[0052] T-butanol solution containing paclitaxel and the lipid fraction were added to the aqueous solution with mixing at 300 rpm in a steady stream in one minute. The weight of lipid fraction (as solution) added was about 15 g.

[0053] The resulting solution, immediately upon completion of t-butanol solution addition was turbid with slight translucence characteristic of liposomes. The temperature of bulk liposome solution immediately after formation was measured to be 36 °C and the solution was mixed for an additional 10 minutes at 300 rpm. The mixing speed was increased to 500 rpm for additional 30 minutes while the bulk liposomes were cooled to 25 °C.

[0054] Liposome size measurement of bulk liposomes showed that they are multi lamellar liposomes with a mean size of 1.3 microns. The pH of bulk liposomes was measured to be 4.63.

[0055] The bulk liposomes were size reduced by extrusion through 0.2μ and 0:1μ pore size polycarbonate membrane filters at 100-200 psi pressure. No drug precipitation was noted during the size reduction process on the filters establishing that the active principal, paclitaxel, is entrapped in the liposomes. Table 2 shows particle size data for liposome based paclitaxel after size reduction. Figure 1 shows the size distribution of size reduced liposomes as measured by dynamic light scattering using PSS instrument. The mean diameter was measured at 120.7 nm (standard deviation = 37.3 nm), and the distribution was as follows: 25% < 86.8 nm, 50% < 107.2 nm, 75% < 132.3 nm, 90% < 158.8 nm, and 99% < 219.7 nm.

Table 2

Liposome size data for bulk liposomes during and after size reduction

Process Step	Measured Size			
	Mean	Standard deviation	D99	Chi^2
Bulk Liposomes	1304 nm	842 nm	4388 nm	121*
After 0.2 μ extrusion	188.4 nm	65.2 nm	372.0 nm	1.44
After 0.1 μ extrusion	120.7 nm	37.3 nm	219.7 nm	2.55

* Chi^2 is too large . Nicomp distribution shows that majoirty ofliposomes are larger than 1μ (1000nm)

[0056] After size reduction, the liposomes were subjected to t-butanol solvent removal using Tangential flow-filtration (TFF) procedure. For this purpose, Pellicon 2 TFF system (Millipore Corp. Bedford, MA) assembled with a 0.1 square meter surface area polyether sulfone (PES) membrane cassette was used. Schematic representation of the TFF system used for freeing the bulk liposomes or size reduced liposomes of t-butanol employed in their formation is shown in Figure 2. The specific membrane cassette used is fabricated with restricted channel screen (type C) capable of retaining any solute molecules (e.g., protein) or organized structures such as liposomes larger than 100,000 Daltons (molecular weight cut-off or MWCO 100 kD) allowing smaller solute molecules (such as sucrose and t-butanol) to pass through the membrane. Size reduced liposomes were first diluted two-fold (2x) by addition of about 200 g of 10% sucrose solution before they are introduced into the TFF system for solvent removal. The inlet flow, inlet pressure, outlet pressure (or back pressure), and filtrate flow were monitored during the process and represented in Table 3. A total of seven iterations (seven volumes of filtrate collected) were performed in concentration-dilution mode of operation.

Table 3

Tangential flow filtration process data for t-butanol solvent removal from liposomes

Liposome Feed Rate (ml/min)	Inlet Pressure (psi)		Wt. of Filtrate Collected (in grams)	Elapsed Time (in minutes)
	Start	End		
400 ml/min	10	11	137 g	5 min
400 ml/min	9	10	209 g	6 min
400 ml/min	8	9	215 g	7 min
400 ml/min	9	9	202 g	7 min
400 ml/min	8	8	236 g	8 min
400 ml/min	8	8	211 g	8 min
400 ml/min	8	9	228 g	10 min

[0057] Solvent removal by tangential flow-filtration process in the case of 500 ml batch was performed in both concentration-dilution mode as well as continuous infusion of aqueous phase as t-butanol containing aqueous phase is removed as filtrate (feed and bleed mode) in two separate experiments. Liposome feed rates up to 600 ml/min were

used which generated higher inlet pressures of up to 20 psi. Large scale process for solvent removal may use up to 100 l/min flow rates that generate inlet pressures of up to 50 psi. TFF membrane cassettes of similar (100 kD), smaller (10 kD) or larger (300 kD) MWCO with larger surface areas (up to 1000 sq. meter) can be used in commercial scale manufacturing to remove t-butanol. However, 100 kD MWCO membrane cassette is the preferred size to be used for the purpose of removing t-butanol from the liposomes. While larger MWCO membrane cassettes such as 300 kD and 500 kD can perform this function, some liposomes will also be lost to the filtrate in the process. The flow chart in Figure 2 can be used for removal of water-miscible organic solvents (t-butanol and ethanol) used in bulk liposome formation from 200 ml scale batches to 200 l scale. While concentration-dilution mode described in the example is practical on small scale (up to 1000 ml), continuous mode (feed and bleed) is practiced on large scale.

[0058] Particle size measurement of liposomes after the solvent removal by TFF process, showed that the liposome size was not affected during the solvent removal process (see Figure 3). The mean diameter was measured at 115.6 nm (standard deviation = 33.6 nm), and the distribution was as follows: 25% < 84.6 nm, 50% < 103.2 nm, 75% < 125.9 nm, 90% < 150.2 nm, and 99% < 202.6 nm.

[0059] Following solvent removal the liposomes were sterile filtered through a MilliPak 20 sterilizing filter before freeze-drying. Sterile filtered liposomes were filled in 20 ml glass (10.5 ml per vial) and freeze dried. Freeze dried liposomes were reconstituted with 10 ml of deionized water. Reconstituted liposomes were analyzed for liposome size, paclitaxel, DOPC, cholesterol, and cardiolipin contents. The size distribution of paclitaxel containing liposomes reconstituted after freeze drying (see Figure 4) did not show any significant changes indicating that liposome integrity is preserved during freeze drying process. The mean diameter was measured at 117.6 nm (standard deviation = 40.2 nm), and the distribution was as follows: 25% < 81.7 nm, 50% < 103.3 nm, 75% < 130.3 nm, 90% < 160.4 nm, and 99% < 230.5 nm.

[0060] These liposomes were also assessed by freeze fracture electron microscopy, using the procedure described above. The electron micrographs obtained show uniform distribution of mostly spherical liposomes of single bilayer (also called small unilamellar vesicles or SUVs for short) with a diameter ranging from 20 to 150 nm. Major composition of the liposomes are individual and not associated or aggregated (see Figure 5).

[0061] The results from analysis of bulk liposomes, after size reduction, sterile-filtration after solvent removal (Table 4) establish that the inventive method can be employed for manufacturing liposomes containing water in-soluble active principals, such as paclitaxel, using t-butanol.

Table 4

Paclitaxel and lipid fraction contents, liposome size results for liposome based paclitaxel formulation prepared using t-butanol

Process Stage	Paclitaxel mg/ml	DOPC mg/ml	Cholesterol mg/ml	Cardiolipin mg/ml	Liposome Size	
					Mean (nm)	D99 (nm)
Target Concentration/ Liposme Size	1.0	27.0	0.75	2.45	100-160	180-250
Bulk Liposomes	1.04	27.5	0.74	2.11	Not applicable	Not applicable
After Size reduction	1.02	27.5	0.74	2.26	120.7	219.7
After solvent removal and before freeze drying	1.05	30.0	0.82	2.18	115.6	202.6
After reconstitution of freeze-dried liposomes	1.05	29.2	0.79	2.34	117.6	230.5

[0062] All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

[0063] While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

What is claimed is:

1. A method of manufacturing a liposomal preparation, said method comprising:

(a) dissolving a lipid fraction in a water-miscible organic solvent,

(b) mixing said water-miscible organic solvent solution comprising said lipid fraction with an aqueous solution under conditions suitable to form a bulk liposomal preparation.

2. The method of claim 1, further comprising adding one or more active principals.

3. The method of claim 2, wherein an active principal is added to said water-miscible organic solvent.

4. The method of claim 3, wherein the active principal is added to the water-miscible organic solvent prior to the addition of the lipid fraction.

5. The method of claim 2, wherein an active principal is added to said aqueous solution.

6. The method of claim 5, wherein the active principal is added to the aqueous solution prior to step (b).

7. The method of claim 2, wherein the active principal is added during or after step (b).

8. A method of manufacturing a liposomal preparation of an active principal, said method comprising:

(a) dissolving at least one active principal in a water-miscible organic solvent,

(b) dissolving a lipid fraction in said water-miscible organic solvent,

(c) mixing said water-miscible organic solvent solution comprising said active principal and said lipid fraction with an aqueous solution under conditions suitable to form a bulk liposomal preparation.

9. The method of any of claims 1-8, wherein the water-miscible organic solvent is t-butanol.

10. The method of any of claims 2-9, wherein the active principal comprises at least one antineoplastic or antifungal agent.

11. The method of any of claims 2-9, wherein the active principal comprises at least one agent selected from the group consisting of taxanes or derivatives and camptothecin or derivatives.

12. The method of any of claims 2-9, wherein the active principal includes paclitaxel or docetaxel

13. The method of any of claims 3-4, wherein the active principal is dissolved in the water-miscible organic solvent at a temperature above about 35 °C.

14. The method of claim 13, wherein the active principal is dissolved in water-miscible organic solvent at a temperature between about 40 °C and about 55 °C.

15. The method of claim 13 or 14, wherein the water-miscible organic solvent is t-butanol.

16. The method of any of claims 1-15, wherein the lipid fraction includes one or more of cholesterol, dioleoylphosphatidylcholine (DOPC), tetramyristoyl cardiolipin, and tocopheryl acid succinate.

17. The method of claim 16, wherein the lipid fraction includes three or more of cholesterol, DOPC, tetramyristoyl cardiolipin, and tocopheryl acid succinate.

18. The method of claim 17, wherein DOPC constitutes a majority of the lipid fraction.

19. The method of claim 18, wherein the lipid fraction comprises at least DOPC, cholesterol, and tetramyristoyl cardiolipin in a molar ratio of about 90:5:5.

20. The method of any of claims 1-19, wherein the aqueous solution is at least about 90% of the volume of the total liposome preparation.

21. The method of any of claims 1-20, wherein the aqueous solution is about 10% sucrose and about 0.4-0.9% sodium chloride.

22. The method of claim 1, wherein step (a) is achieved by addition of the active principal, paclitaxel, or any other taxane such as docetaxel, to the water-miscible organic solvent at about 35 to about 65 °C.

23. The method of claim 1, wherein step (a) is achieved by sequential addition of the lipid components, cholesterol, cardiolipin, DOPC, and tocopheryl acid succinate, that comprise the lipid fraction into the water-miscible organic solvent at about 35 to about 65 °C

24. The method of claim 8, wherein step (b) is achieved by sequential addition of the compounds that comprise the lipid fraction into the water-miscible organic solvent.

25. The method of claim 1, wherein step (b) involves adding the water-miscible organic solvent solution comprising the lipid fraction to the aqueous solution while maintaining the aqueous phase solution at about 30-40 °C and mixing at 300-400 rpm.

26. The method of claim 8, wherein step (c) involves adding the water-miscible organic solvent solution comprising the lipid fraction to the aqueous solution while mixing.

27. The method of claim 1, wherein step (b) involves mixing of solution following addition of water-miscible solvent comprising the lipid fraction to the aqueous solution while cooling.

28. The method of claim 8, wherein step (c) involves mixing of solution following addition of water-miscible solvent comprising the lipid fraction to the aqueous solution while cooling.

29. The method of claims 27 or 28, wherein the cooling is to a temperature between about 25 °C and about 30 °C.

30. The method of any of claims 1-29, further comprising size-reducing the bulk liposomal preparation to obtain a size-reduced liposomal preparation.

31. The method of claim 30, wherein said size-reduction is achieved by extrusion of the bulk liposomal preparation through polycarbonate filters.

32. The method of claim 31, wherein size-reduction is achieved by extrusion of the bulk liposomal preparation through 0.2 μm and 0.1 μm polycarbonate filters.

33. The method of claim 31 or 32, wherein size-reduction is achieved by extrusion of the bulk liposomal preparation at pressures up to about 200 psi.

34. The method of any of claims 31-33, wherein the bulk liposomal prepration comprises at least one active principal.

35. The method of claim 34, wherein size reduction is achieved without precipitation of the active principal.

36. The method of any of claims 1-35, further comprising substantially freeing the liposomal preparation of the water-miscible organic solvent.

37. The method of any of claims 30-35, further comprising substantially freeing the liposomal preparation of the water-miscible organic solvent.

38. The method of claim 36 or 37, wherein the liposomal preparation is substantially freed of water-miscible organic solvent by diafiltration using a tangential flow filtration process and sterile filtration.

39. The method of any of claims 36-38, further comprising adding one or more active principals.

40. The method of claim 39, wherein the active principal is a water-soluble principle, which is added after the liposomal preparation is substantially freed of the water miscible organic solvent.

41. The method of any of claims 1-40, further comprising sterile-filtering said liposomal preparation.

42. The method of claim 41, wherein an active principal is added to the formulation prior to sterile filtration.

43. The method of any of claims 1-42, further comprising freeze-drying said liposomal preparation.

44. The method of claim 43, wherein an active principal is added to the formulation prior to freeze-drying said liposomal preparation.

45. A liposomal preparation produced by the process of any of claims 1-37.

46. The liposomal preparation of claim 45, comprising at least one active principal.

47. The liposomal preparation of claim 46, wherein an active principal comprises at least one antineoplastic or antifungal agent.

48. The liposomal preparation of claim 46, wherein an active principal comprises at least one agent selected from the group consisting of taxanes or derivatives and camptothecin or derivatives.

49. The liposomal preparation of claim 46, wherein an active principal wherein the active principal comprises paclitaxel or docetaxel.

50. The liposomal preparation of any of claims 45-49, further comprising a pharmaceutically-acceptable excipient.

51. A method of treating a disease in patient in need of treatment, comprising administering to the patient a composition according to any of claims

47-50, in amounts and at a location sufficient to deliver said active agent to the patient so as to treat said disease.

52. The method of claim 51, wherein said disease is cancer.

53. The method of claim 51 or 52, wherein said composition is administered parenterally.

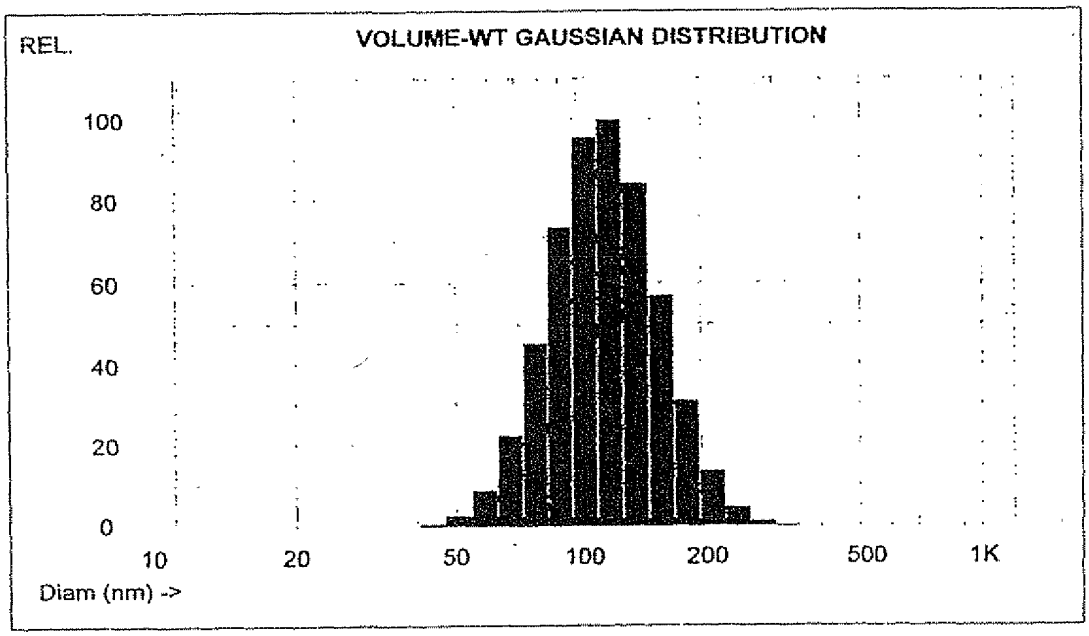
54. The method of claim 51 or 52, wherein said composition is administered topically.

55. The method of claim 51 or 52, wherein said composition is administered by direct injection into a tumor.

VOLUME-Weighted GAUSSIAN DISTRIBUTION Analysis (Vesicle)

GAUSSIAN SUMMARY:

Mean Diameter	= 120.7 nm	Chi Squared	= 2.550
Stnd. Deviation	= 37.3 nm (30.9 %)	Baseline Adj.	= 0.000 %
Coeff. of Var'n	= 0.309	Mean Diff. Coeff.	= 3.85E-008 cm2/s



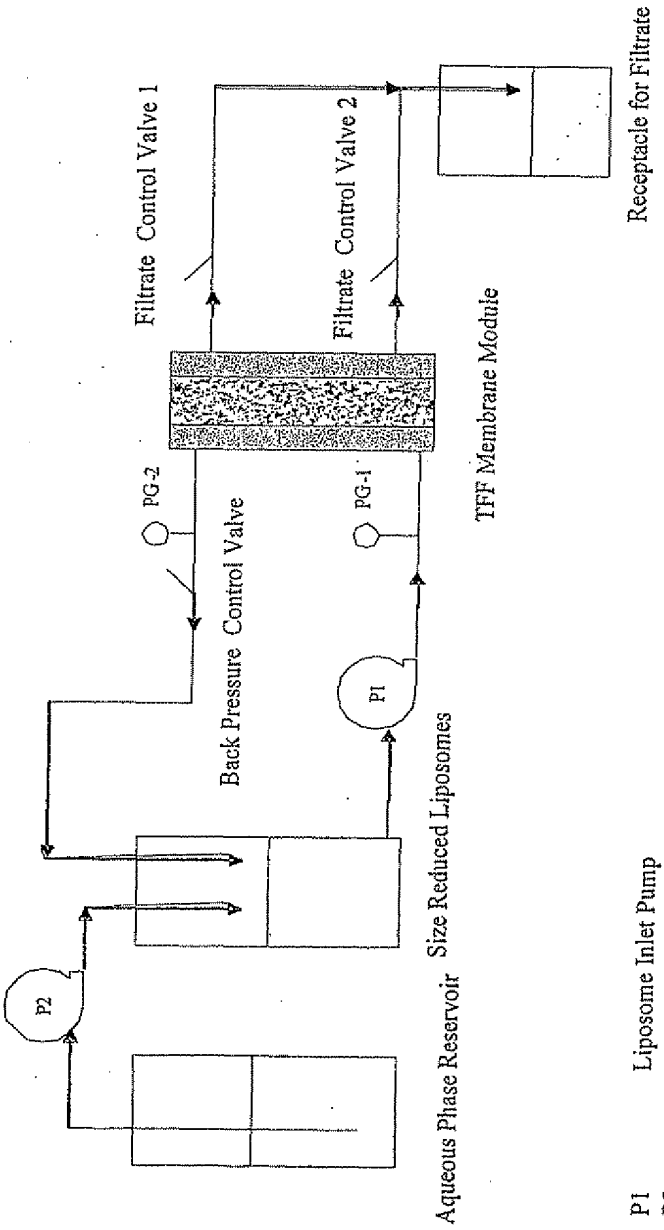
Cumulative Result:

25 % of distribution <	86.8 nm
50 % of distribution <	107.2 nm
75 % of distribution <	132.3 nm
90 % of distribution <	158.8 nm
99 % of distribution <	219.7 nm

Run Time	= 0 Hr 15 Min 21 Sec		Wavelength	= 632.8	nm
Count Rate	= 298	KHz	Temperature	= 23	deg C
Channel #1	= 761.4	K	Viscosity	= 0.933	cp
Channel Width	= 14.0	uSec	Index of Ref.	= 1.333	

Figure 1

FLOW CHART FOR SOLVENT REMOVAL BY TANGENTIAL FLOW FILTRATION



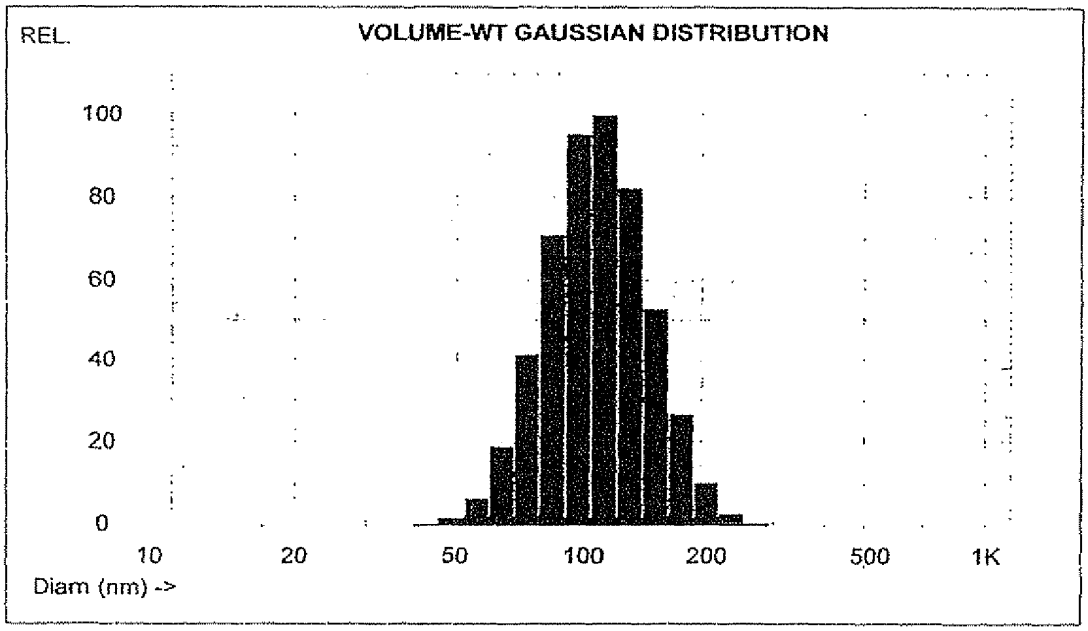
P1 Liposome Inlet Pump
P2 Aqueous Phase Feed Pump
PG-1 Inlet Pressure gauge on E-01
PG-2 Outlet Pressure gauge

Figure 2

VOLUME-Weighted GAUSSIAN DISTRIBUTION Analysis (Vesicle)

GAUSSIAN SUMMARY:

Mean Diameter	= 115.6 nm	Chi Squared	= 1.346
Stnd. Deviation	= 33.6 nm (29.1 %)	Baseline Adj.	= 0.000 %
Coeff. of Var'n	= 0.291	Mean Diff. Coeff.	= 4.02E-008 cm2/s



Cumulative Result:

25 % of distribution <	84.6 nm
50 % of distribution <	103.2 nm
75 % of distribution <	125.9 nm
90 % of distribution <	150.2 nm
99 % of distribution <	202.6 nm

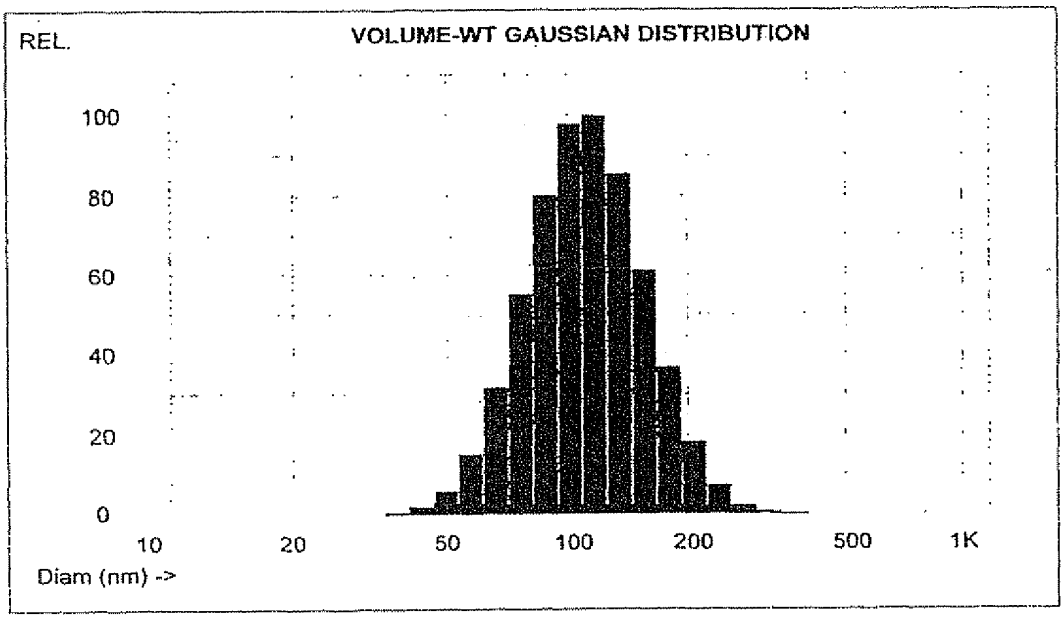
Run Time	= 0 Hr 15 Min 21 Sec	Wavelength	= 632.8 nm
Count Rate	= 329 Kiz	Temperature	= 23 deg C
Channel #1	= 863.5 K	Viscosity	= 0.933 cp
Channel Width	= 13.0 uSec	Index of Ref.	= 1.333

Figure 3

VOLUME-Weighted GAUSSIAN DISTRIBUTION Analysis (Vesicle)

GAUSSIAN SUMMARY:

Mean Diameter	= 117.6 nm	Chi Squared	= 1.657
Stnd. Deviation	= 40.2 nm (34.2 %)	Baseline Adj.	= 0.000 %
Coeff. of Var'n	= 0.342	Mean Diff. Coeff.	= 3.95E-008 cm2/s



Cumulative Result:

25 % of distribution <	81.7 nm	
50 % of distribution <	103.3 nm	
75 % of distribution <	130.3 nm	
90 % of distribution <	160.4 nm	
99 % of distribution <	230.5 nm	

Run Time	= 0 Hr 15 Min 25 Sec	Wavelength	= 632.8 nm
Count Rate	= 292 KHz	Temperature	= 23 deg C
Channel #1	= 755.7 K	Viscosity	= 0.933 cp
Channel Width	= 14.0 uSec	Index of Ref.	= 1.333

Figure 4

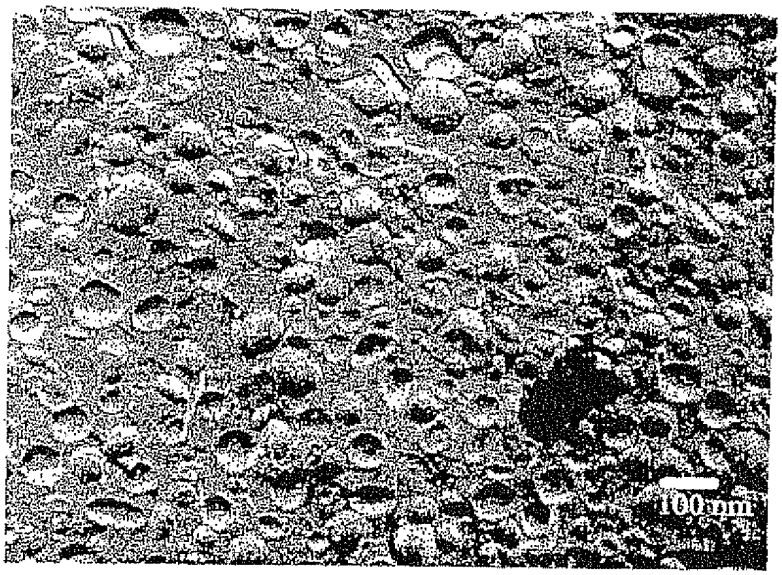


Figure 5

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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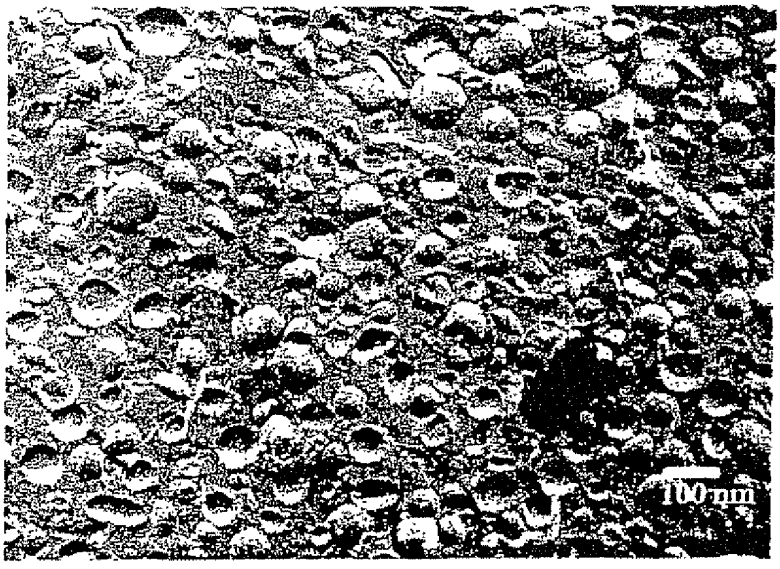
2 December 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MANUFACTURING PROCESS FOR LIPOSOMAL PREPARATIONS



(57) Abstract: The present invention provides manufacturing processes for liposomal preparations. In accordance with the methods, a lipid fraction is dissolved in a water-miscible organic solvent. This solution comprising the lipid fraction can be added to and mixed with an aqueous solution under conditions to form a bulk liposomal preparation. Desirably, the preparation can include one or more active principals. The bulk liposomal preparation can be further processed as desired, for example, by size fractionation or reduction, removal of the water-miscible organic solvent, freeze-drying, or other treatment. The methods permit the production of liposomal formulations on a large or commercial scale.

WO 2004/071466 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/004555

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K31/337 A61K31/4745

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 750 910 A (YOSHITOMI PHARMACEUTICAL) 2 January 1997 (1997-01-02) abstract examples 1-4	1-10, 13-17, 20,21, 23,26, 30,36-50
X	US 6 066 331 A (BARENHOLZ YECHEZKEL ET AL) 23 May 2000 (2000-05-23) abstract examples 1,6	1-10, 13-17, 20,21, 23,26, 30,36-50

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 September 2004	13/09/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Felder, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/004555

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/70220 A (NEOPHARM INC ; RAHMAN AQUILAR (US)) 27 September 2001 (2001-09-27) the whole document	45-50
X	WO 01/56548 A (ZADI BRAHIM ; LIPOXEN TECHNOLOGIES LTD (GB)) 9 August 2001 (2001-08-09) the whole document	45-50
X	LUNDBERG B B: "BIOLOGICALLY ACTIVE CAMPTOTHECIN DERIVATIVES FOR INCORPORATION INTO LIPOSOME BILAYERS AND LIPID EMULSIONS" ANTI-CANCER DRUG DESIGN, BASINGSTOKE, GB, vol. 13, no. 5, 1998, pages 453-461, XP000878681 ISSN: 0266-9536 the whole document	45-50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/004555

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 51-55 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US2004/004555

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